Circulating T cells in patients with untreated acute myelogenous leukemia are heterogeneous and can be activated through the CD3/TCR complex
Circulating T cells in patients with untreated acute myelogenous leukemia are heterogeneous and can be activated through the CD3/TCR complex

ELISABETH ERSVÆR1, PETER HAMPSON2, ØYSTEIN WENDELBO1,3, JANET M LORD2, BJØRN TORE GJERTSEN1, & ØYSTEIN BRUSERUD1

1University of Bergen and Haukeland University Hospital, Institute of Medicine, Bergen, Norway, 2MRC Centre for Immune Regulation, University of Birmingham, Birmingham, UK, and 3Division for Infectious Diseases, University of Bergen and Haukeland University Hospital, Bergen, Norway

(Received 9 October 2006; accepted 15 January 2007)

Abstract
T lymphocyte defects may contribute to the immune insufficiency seen in acute myelogenous leukemia (AML). We therefore characterized the T cell system for untreated AML patients. T lymphocyte subsets were analyzed by flow cytometry for 45 AML patients. The in vitro interferon-γ (IFNγ) release in response to stimulation with anti-CD3 plus anti-CD28 in the presence of autologous AML cells was examined for 31 patients. The majority of circulating lymphocytes were CD3+ T cells, and CD19+ B cells usually constituted 10% of the lymphocytes. Most T cells expressed the αβ T cell receptor (TCRαβ+), and only a minority of the cells was TCRγδ+. Both CD4+ and CD8+ T cells were detected, the CD4:CD8 ratio showed a wide variation but was generally >1.0. The majority of CD4+ and CD8+ T cells were CD45RA+ cells. The T cells could be stimulated to release IFNγ in response to anti-CD3 plus anti-CD28 ligation even in the presence of excess autologous AML blasts, and for a subset of patients (6 of 27) these IFNγ levels could be further increased by the novel protein kinase C (PKC) agonist PEP005. In conclusion, circulating T cells in patients with untreated AML are mainly CD4+ or CD8+ TCRαβ+; both CD45RA+ and CD45R0+ cells can be detected, and these cells can be activated through the CD3/TCR complex even in the presence of excess AML cells. For a subset of patients T cell responsiveness can be further increased by targeting PKC and these data therefore suggest that T cell function is not inhibited in AML patients.

Keywords: Cytopenia, T lymphocytes, chemotherapy, cytokines, AML

Introduction
Acute myelogenous leukemia (AML) is an aggressive malignancy characterized by the accumulation of immature leukemia cells in the bone marrow [1]. Intensive chemotherapy, eventually followed by hematopoietic stem cell transplantation, is the only possible cure for the disease. This treatment is followed by severe therapy-induced pancytopenia that often lasts for up to 3 weeks before hematopoietic and lymphoid reconstitution occurs [2–6].

Previous clinical studies have focused on the time until hematopoietic reconstitution after intensive AML chemotherapy because the treatment-related mortality is mainly due to neutropenia-associated infections or severe bleeding due to thrombocytopenia [1]. However, several recent studies have reported that the time until lymphoid reconstitution has a prognostic impact in several malignancies, and late reconstitution is generally associated with an increased risk of early progression or relapse [7–12]. This is also true for AML patients receiving conventional chemotherapy as well as high-dose therapy followed by autologous hematopoietic stem cell rescue. One recent study of AML patients receiving conventional therapy included 103 patients and reported that early post-chemotherapy lymphocyte recovery was an independent prognostic parameter in AML and associated
with increased overall as well as AML-free survival [8]. These observations raise the question of whether the pre-therapy status of the cellular immune system in AML patients influences lymphoid reconstitution and thereby the outcome for the patient.

Previous studies of the T cell system in patients with untreated AML have included relatively few patients and have reported that the absolute numbers of CD4$^{+}$ and CD8$^{+}$T cells do not differ significantly from healthy controls, but the patients showed a wider variation for both cell subsets [13]. More detailed T cell subset characterization (e.g. TCR subsets, CD45 isoform expression) together with functional investigations have not been performed in untreated AML. In the present study, we therefore characterized the T cell system for a large group of AML patients, and we included T cell subset analysis as well as functional and pharmacological studies. Our observations suggest that the status of the cellular immune system in advanced AML is functional, and that further studies may be feasible to determine the impact of the status of the immune system for overall survival.

Materials and methods

Patients

The studies were approved by the local Ethics Committee and blood samples collected after informed consent in accordance with the Declaration of Helsinki. We used density gradient separation (Lymphoprep, NyCoMed, Oslo, Norway, specific density 1.077) to prepare leukemic peripheral blood mononuclear cells (PBMC). Our study included patients with relatively high peripheral blood blast counts, and by using this methodological approach the leukemic PBMC will contain a majority of AML blasts (at least 95%) together with a smaller subset of normal PBMC (mainly lymphocytes) [14]. These frequencies of normal and leukemic cells were based on morphological and flow-cytometric studies [14]. Quantitative flow-cytometric studies (50 patients examined, a T cell population detected for 45 patients) and functional/pharmacological T cell studies (31 patients) were performed in two different and partially overlapping consecutive groups (Table I). Normal PBMC were derived from age matched healthy adult volunteers. Both normal and leukemic PBMC were stored frozen in liquid nitrogen until analysed [14].

Quantitative analysis of lymphocyte subsets by flow cytometry

A four-colour flow cytometry (FCM) analysis was performed using a FACS Calibur System (Becton-Dickinson, Mountain View, CA, USA).

<table>
<thead>
<tr>
<th>AML patients/characteristic</th>
<th>Flow cytometry study (number/values)</th>
<th>IFN$\gamma$ release study (number/values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients included</td>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td>Age; median (range)</td>
<td>62 (29–82)</td>
<td>63 (29–82)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>24/21</td>
<td>14/17</td>
</tr>
<tr>
<td>Predisposition/previous disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous chemotherapy</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Previous myelodysplasia or chronic myeloproliferative disease</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>AML relapse</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>No predisposition</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>FAB classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0/M1</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>M2/M3</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>M4/M5</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>M6/M7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytogenetic abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favourable</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Normal/intermediate risk</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>High risk</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Not examined</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Genetic Flt3 abnormalities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal tandem duplication</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>D835 point mutation</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>Not examined</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Number of CD34$^{+}$ samples</td>
<td>28</td>
<td>21</td>
</tr>
</tbody>
</table>

We investigated two partly overlapping consecutive patient groups. Fifty patients were examined by flow cytometry, but a lymphocyte population was identified only for those 45 patients described in the table. CD34-positivity was defined as at least 20% positive cells compared with an isotypic control. The cytogenetic abnormalities were classified according to the MRC criteria [33]: (i) Favourable including inv(16), t(8;21), t(15;17); (ii) high risk including, del(5q), −7, inv(3)/t(3;3), −5, complex; (iii) intermediate, other abnormalities; and (iv) normal karyotype.
Lymphocyte and leukemic blast gates were set according to forward (FSC) and side scatter (SSC) (both acquired in linear mode) together with CD3 and TCRαβ expression. Each analysis was based on 30,000 total events and data were analyzed using FlowJo software (Tree Star, Inc., OR, USA). The following anti-human monoclonal antibodies were used: FITC-conjugated anti-CD8 and TCR(both acquired in linear mode) together with CD3 and APC-conjugated anti-CD56, anti-CD45-RO and anti-CD14 (Becton Dickinson, San Jose, CA, USA). The cut-off for positive cells was defined as fluorescence corresponding to 1% positive cells when using isotypic control antibodies.

In vitro culture and activation of lymphocytes

Reagents. X-vivo 10™ culture medium (BioWhittaker, Walkersville, MA, USA) with 100 μg/ml of gentamicin was used [15]. Anti-CD3 (1.5 mg/ml; mouse IgE Moab; CLB-T3/4.E) and anti-CD28 (2 mg/ml; mouse IgG1 Moab, CLB-CD28/1) were purchased from The Central Laboratory of the Netherlands Red Cross Blood Transfusion Services (Amsterdam, The Netherlands). Antibodies were diluted in culture medium (0.1 ml antibody diluted up to 12.5 ml, final dilution 1:125) [16], and these stock solutions were stored at −70°C. The anti-CD3 Moab was further diluted to 1:500 and anti-CD28 to 1:250 before use in the experiments (both Moabs then added at 20 μl/well, see below). The use of these concentrations was based on previous experimental data together with recommendations from the distributor [3,15,16]. PEP005 was a generous gift from Peplin Ltd (Brisbane, Australia), and based on previous observations the drug was tested at 10 nM. Firstly, in pilot experiments T cells derived from 5 healthy individuals and 4 leukemia patients with chemotherapy-induced cytopenia were stimulated with anti-CD3 + anti-CD28 + exogenous IL2 in a whole blood assay [15], and a dose-dependent increase in T cell proliferation was then observed and Pep005 10 nM caused a strong enhancement of the T cell proliferation. Secondly, even though Pep005 10 nM increases in vitro AML cell apoptosis, a subset of viable AML cells can be detected after 2 days of culture and then function as accessory cells during T cell activation (Hampson, unpublished data). Finally, the concentration 10 nM has been used in previous studies and is known to induce PKC activation [17].

In vitro lymphocyte activation. Our assay for in vitro T cell activation was based on the whole-blood proliferation assay described previously by Wendelbo et al. [3,15], the only exception being that cells were added as a suspension of gradient-separated PBMC instead of diluted whole blood. Cells were cultured in U-bottomed microtiter wells (Costar 3790, Corning Incorp., NY, USA), and into each well were then distributed: (i) 50 μl of PBMC 5 × 10⁴ cells in culture medium; (ii) 100 μl of medium; (iii) 20 μl of anti-CD3 plus 20 μl of anti-CD28. Supernatants were harvested after 4 days of incubation at 37°C in a humidified atmosphere of 5% CO₂.

Analysis of interferon-γ (IFNγ) levels. IFNγ levels were determined by ELISA analysis (Quantikine ELISA kits; R&D Systems, Abingdon, UK). All analyses were performed strictly according to the manufacturer’s instructions. Standard curves were constructed by using the mean of duplicate determinations, and differences between duplicates were generally <10% of the mean. The minimum detectable level was 8 pg/ml.

Presentation and analysis of the data

To avoid any influence of contaminating non-lymphocytic cells within the lymphocyte gate we present our data as ratios between various cell subset. This presentation allows us to identify the dominating cell populations. In addition we present our results as percentages of positive cells in the text. The Wilcoxon’s test for paired samples (rank sum test) or Mann–Whitney U was used for statistical comparisons. Differences were regarded as statistically significant when p < 0.05.

Results

Characterization of the lymphocyte subpopulations in patients with untreated AML

Gating of lymphocytes was based on flow cytometric FSC and SSC and expression of the T lymphocyte markers CD3 and TCRαβ. The lymphocyte population could be identified either as a separate population in the forward–side scatter diagram or the lymphocytes showed partial or complete overlap with the dominating AML cell population when using anti-CD3 and anti-TCRαβ for identification (Figure 1). Throughout our data presentation we describe the results both for our whole study population (45 patients, see below) and for those patients with non-overlapping cell populations; this is to document that the results for the whole patient populations are reliable and comparable with the results for patients showing separated AML and T cell populations.

A total of 50 unselected patients were examined. Most patients with complete overlap were AML-M0/M1 with high expression of CD34 compared with the patients without overlapping populations (data not shown). For a minority of
patients no lymphocyte population could be identified (5 patients, corresponding to 10%) and these patients were not included in subsequent analyses. Two patients with overlapping AML and lymphocyte populations showed aberrant CD19-expression and were therefore also excluded from this part of the analysis (see below).

We first determined the ratio between CD19⁺ B lymphocytes and CD3⁺ T lymphocytes (Figure 2). The AML cells outside the lymphocyte gate were negative for the two markers compared with the isotype controls (43 patients see above). For the majority of patients most circulating lymphocytes were CD3⁺ T lymphocytes (CD19:CD3 ratio < 1; see Figure 2). As a control we analysed separately those patients showing no overlap between lymphocytes and AML cells in the FSC/SSC analysis (Figure 1), and a similar dominance of T lymphocytes was detected for these patients (n = 27, median ratio 0.06, range 0.01–1.5) as for the whole study population of 43 patients. Thus, the B lymphocytes constituted only a small subset of the circulating lymphocytes both when analysing the whole study population (n = 43, median 0%, range 0–18%) and when analysing patients with non-overlapping AML and lymphocyte populations (n = 27, median 3%, range 0–18%).

We investigated the TCRγδ/TCRαβ ratio and for most patients the dominant T cell population was TCRαβ⁺ cells (TCRγδ/TCRαβ ratio < 1, see Figure 2). A similar dominance of TCRαβ⁺ cells was observed when only analyzing patients without overlap between lymphocyte and leukemia cell populations (median ratio 0.04, range 0.01–0.3). Furthermore, the dominance of TCRαβ⁺ cells is also illustrated by the observation that TCRγδ⁺ T cells was

Figure 1. Flow cytometric identification and analysis of the lymphocyte population in leukemic PBMC. (A–C) show either separate (A), partly overlapping (B) or overlapping (C) T lymphocyte and AML blast populations. The lymphocyte population of PBMC derived from the untreated AML patients were identified by the FSC and SSC as well as positive staining for the T lymphocyte markers CD3 and TCRαβ. The markers CD3 (middle column) and TCRαβ (right column) were used to backgate and to locate the lymphocytes in the scatter diagram (left column). Gate 1 indicates the AML blasts and Gate 2 indicates the lymphocytes. Results are for three representative patient samples.
very low both for the whole study population \((n = 45, \text{median } 0\%, \text{range } 0–17\%)\) and for patients with non-overlapping cell populations \((n = 27, \text{median } 0\%, \text{range } 0–17\%)\).

The ratio between CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) cells exceeded 1 for the majority of patients both when investigating the overall patient population (Figure 2) and those patients without overlap between lymphocytes and AML cells (median ratio 1.9, range 0.4–2.4; median percentages of CD3\(^+\)CD4\(^+\)57\% (range 25–87) and CD3\(^+\)CD8\(^+\)31\% (range 7–68) of CD3\(^+\) cells). This was not significantly different from a group of 9 healthy age matched controls (Table II). CD4/CD8 double positive and double negative lymphocytes constituted a minority (<2\% of the lymphocytes) for all patients and controls, but all CD4\(^+\) and CD8\(^+\) cells were also CD3\(^+\)(>95\% for all patients). Finally, a dominance of CD4\(^+\) vs. CD8\(^+\) cells within the CD3\(^+\) lymphocyte population was also detected when comparing the percentages of positive cells for the whole study population (median percentages of positive cells 57 vs. 29\%) and for patients with non-overlapping AML and lymphocyte populations (median values 57 vs. 31\%).

Only a minority of the patients’ CD3\(^+\)T lymphocytes expressed CD56 (<3\% for all 45 patients examined). Furthermore, only a minor subset of circulating lymphocytes (i.e. cells within the lymphocyte gate) were CD3\(^+\)CD56\(^+\) NK cells \((n = 45, \text{median } 3\%, \text{range } 0–32\%)\).

Expression of CD45RA and CD45RO was compared for CD4\(^+\)T cells. All CD4\(^+\) cells were CD3\(^+\) (see above). The median CD45RA/CD45RO ratio for the patient CD4\(^+\) cells was 4.1, and an excess of CD4RA\(^+\) cells was detected both when analyzing all patients (median 4.1, range, 0.2–96) and only those patients with separated lymphocyte and AML blast populations (median ratio 4.1, range 0.6–96). This did not differ from healthy controls. The overall results reflect that there was a considerable difference between patients with regard to the percentage of CD45RA\(^+\) \((n = 45, \text{median } 38\%, \text{range } 0–100\%)\), CD45RO\(^+\) cells (median 6\%, range 0–65\%) and double positive cells (median 0\%, range 0–32\%) among the CD4\(^+\) cells.

Table II. A comparison of quantitative and qualitative T cell characteristics for circulating T cells derived from patients with untreated AML and healthy controls.

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>AML patients (&lt;2500 T cells)</th>
<th>Controls (~30,000 T cells)</th>
<th>Controls (~2500 T cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4/CD8 ratio</td>
<td>1.7 (0.01–12.4)</td>
<td>2.0 (1.0–6.9)</td>
<td>–</td>
</tr>
<tr>
<td>CD4* CD45RA/RO ratio</td>
<td>4.1 (0.2–96)</td>
<td>4.6 (1.4–64)</td>
<td>–</td>
</tr>
<tr>
<td>CD8* CD45RA/RO ratio</td>
<td>2.3 (0.4–68)</td>
<td>15 (1.5–37)*</td>
<td>–</td>
</tr>
<tr>
<td>IFNy release*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous release</td>
<td>&lt;8 (&lt;8–2478)</td>
<td>&lt;8 (&lt;8–&lt;8)**</td>
<td>&lt;8 (&lt;8–&lt;8)***</td>
</tr>
<tr>
<td>aCD3 + aCD28</td>
<td>1856 (&lt;8–124,592)</td>
<td>31,658 (3224–51,230)**</td>
<td>2638 (269–4270)</td>
</tr>
<tr>
<td>aCD3 + aCD28 + PEP005</td>
<td>808 (&lt;8–77,746)</td>
<td>9142 (794–16,330)*</td>
<td>762 (66–1361)</td>
</tr>
</tbody>
</table>

* We cultured \(5 \times 10^6\) normal and leukemic PBMC per well. The T cells in controls were present at ~60\%, equivalent to \(3 \times 10^6\) cells/well. This column presents the IFNy values adjusted for ~2500 T cells, equal to the T cell numbers for the AML patients; ‡ Lymphocyte subsets were analyzed by flow cytometry and the results for 45 patients were compared with the results for 9 healthy controls (3 men and 6 women; median age 56 years with variation range 30–64 years). The results are presented as the median and the variation range; § Spontaneous and anti-CD3 + anti CD28 (aCD3 + aCD28) stimulated IFNy release was investigated for 31 patients, the effect of PEP005 was examined for 27 patients. These results were compared with a group of 7 healthy controls (3 men and 4 women; median age 54 years, range 40–66 years). IFNy levels in culture supernatants are presented as median pg/ml (range) (*, \(p \leq 0.01\); **, \(p \leq 0.001\); ***, \(p \leq 0.001\)).

Figure 2. T cell subsets in patients with untreated AML. The figure presents the ratio of CD19+ B cells vs. CD3+ T cells (CD19:CD3 ratio, left part), TCR\(\gamma\delta\) vs. TCR\(\alpha\beta\)+ T cells (TCR\(\alpha\beta\):TCR\(\gamma\delta\), middle) and CD3+CD8+ vs. CD3+CD4+ T cells (CD8:CD4 ratio, right part). The data are derived from analysis of 43 patient samples.
Expression of CD45RA and CD45RO was then examined for the CD8\(^+\) T cells. An excess of CD45RA\(^+\) cells was also seen for the CD8\(^+\) subset. The median CD45RA/CD45RO ratio for the CD8\(^+\) cells was 2.3 (range 0.4–68) when investigating all patients, and similar values were also observed when patients without overlapping AML cell/lymphocyte populations were analysed separately (median ratio 2.8, range 1.1–8). However, the two populations differed and in contrast to the CD4\(^+\) cells the CD45RA/RO ratio for the CD8\(^+\) patient cells was significantly lower than for the healthy controls (\(p < 0.002\); Table II). When the percentages of CD45RA\(^+\) and CD45RO\(^+\) cells were compared there was a considerable variation between the patients (\(n = 45\)) in the frequency of CD45RA\(^+\) cells (median 46%, range 0–100%), CD45RO\(^+\) cells (median 12%, range 0–73%) and double positive cells (median 0%, range 0–73%). Thus, there is a considerable variation between patients in the expression of CD45RA and CD45RO both among CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) T lymphocytes.

**Activation of normal T cells among leukemic PBMC**

The gradient-separated leukemic PBMC contained a majority of AML cells (>95%) and a minor population of remaining normal cells mainly consisting of small lymphocytes, as judged by light microscopy. It has become clear in recent years that the bone marrow can function as a secondary lymphoid organ, providing a significant microenvironment for priming of T cell responses (18). As AML is a disease of the bone marrow we felt it was important, having established that T cell subset numbers were not grossly affected in AML, to investigate whether T cells could be activated within this microenvironment in the presence of a dominating AML blast population. We used anti-CD3 plus anti-CD28 as the T cell stimulatory signal, and T cell activation was measured as IFN\(\gamma\) release. Detectable IFN\(\gamma\) levels were observed for 7 out of the 31 samples when leukemic PBMC were cultured in medium alone, but these levels were usually low and exceeded 100 pg/ml only for 2 patients (Figure 3). As AML blasts are also known to produce IFN\(\gamma\) (19), it is possible that this resting level of cytokine was derived in part from the AML blasts themselves. T cells could be activated to release IFN\(\gamma\) for 29/31 patients (Figure 3; Wilcoxon’s test for paired samples, \(p < 0.0001\)). The activation-dependent IFN\(\gamma\) increase was reproduced in independent experiments for 12 unselected patients (data not shown). IFN\(\gamma\) levels showed a very wide variation that was independent of AML cell morphology (FAB classification), expression of the CD34 stem cell marker or genetic abnormalities (cytogenetic abnormalities, FLT3 and NPM1 mutations) (data not shown). The results for the AML patients were compared with PBMC from 7 healthy age matched individuals (Table II). We cultured 5 × 10\(^4\) normal PBMC per well, the same number used for the leukemic PBMC. For the leukemic PBMC we cultured less than 5% nonleukemic cells and were cultured in vitro in either medium alone, medium with anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD28 plus 10 nM PEP005. Supernatants were harvested after 4 days and IFN\(\gamma\) levels determined by ELISA analysis. The figure compares IFN\(\gamma\) levels presented in a dot plot where each circle represents one sample. The median is denoted with a line. Open circles are samples with IFN\(\gamma\) concentration below the minimal detectable level of 8 pg/ml.

![Figure 3. Release of IFN\(\gamma\) by circulating T cells derived from patients with untreated AML. Leukemic PBMC contained less than 5% nonleukemic cells and were cultured in vitro in either medium alone, medium with anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD28 plus 10 nM PEP005. Supernatants were harvested after 4 days and IFN\(\gamma\) levels determined by ELISA analysis. The figure compares IFN\(\gamma\) levels presented in a dot plot where each circle represents one sample. The median is denoted with a line. Open circles are samples with IFN\(\gamma\) concentration below the minimal detectable level of 8 pg/ml.](image-url)
responsiveness of T cells was not modified in the presence of the AML cells.

The effect of PKC activation on cytokine release by anti-CD3 plus anti-CD28 stimulated T cells derived from patients with untreated AML

Although activation of T cells was not modified on a per cell basis, the magnitude of T cell responses was affected by the reduced overall numbers of T cells and it was important to determine if this could be overcome. We investigated the effect of the novel protein kinase C (PKC)-activator PEP005 (10 nM) on IFNγ release for normal PBMC and leukemic samples. PEP005 has been reported recently to induce apoptosis in AML cells in the nanomolar range, mediated by activation of PKC-δ [17]. As this agent is in pre-clinical development for treatment of AML, and activators of PKC such as PMA are known to be able to activate T cells, we determined whether PEP005 could potentiate the activation of normal T cells in AML patients. PEP005 alone caused only a weak activation of IFNγ release by normal PBMC derived from 6 healthy individuals. All levels were <8 pg/ml when incubated in medium alone and in the presence of PEP005 the median level was 412 pg/ml (range <8–1228 pg/ml). This median level corresponds to <2% of the corresponding level for anti-CD3 + anti-CD28 stimulated control cultures (see above). Low IFNγ levels in the presence of PEP005 alone were also observed for AML samples (data not shown). We then investigated the effect of PEP005 in the presence of anti-CD3 and anti-CD28. IFNγ levels were compared for leukemic PBMC (n = 27) activated by anti-CD3 plus anti-CD28 in medium alone and in the presence of PEP005 (10 nM) (Figure 3 and Table II). PEP005 had divergent effects on IFN-γ release, for healthy PBMC PEP005 slightly reduced IFNγ production when compared to anti-CD3 plus anti-CD28 alone, though the effect was not statistically significant (p = 0.16). For AML derived PBMC there was a modest reduction which was statistically significant overall (p < 0.02). However, for a subset of 6 of the 27 patients tested a median increase of 157% of the IFNγ levels was detected in the presence of PEP005. Thus, pharmacological agents such as PEP005 may be able to improve the T cell cytokine responsiveness of a subset of the AML patients.

Discussion

Early lymphoid reconstitution, most probably T cell reconstitution, after intensive chemotherapy for AML seems to have a prognostic impact both for patients receiving conventional chemotherapy and high-dose therapy with autologous stem cell rescue [8,9,18]. Post-chemotherapy T cell reconstitution will be determined both by disease-induced pre-therapy alterations and chemotherapy-induced effects. Several previous reports have characterized early and late treatment-induced T cell abnormalities [19–22], but very few studies have investigated the T cell system in patients with untreated AML [13,23–25]. In our present study we describe that these patients have a residual T cell system that can be activated in a microenvironment dominated by AML cells, though these immune responses were significantly lower than for AML derived PBMC. However once data were adjusted for reduced T cell numbers in the AML PBMC the T cell responses were equivalent. Attempts to overcome the diminished overall T cell response with a novel PKC activator PEP005 were only successful in a subset (6 out of 27) AML patient samples.

In our study of patients with untreated AML we used gradient-separated PBMC that include a majority of primary AML cells and a minor component of normal immunocompetent cells (<5% non leukemic cells, mainly small lymphocytes [14]). We used this simple approach for cell preparation to minimize the risk of procedure-induced alteration of immunocompetent and leukemic cells. We also wanted to investigate the functional and phenotypic characteristics of these cells in a natural microenvironment dominated by AML cells. The latter is very relevant to T cell function in AML as it has now become clear that the bone marrow is not simply a primary lymphoid organ producing lymphocyte precursors and mature B cells, but is also a major secondary lymphoid organ. Feuerer et al. showed that naive T cells can home to the bone marrow as a part of their normal recirculatory pathway and become primed by CD11c+ dendritic cells resident in the stroma to generate primary and memory T cell responses [26]. As the bone marrow of patients with AML will be severely compromised by the presence of large numbers of AML blasts, it is possible that reduced T cell responsiveness in this environment could contribute to the immunodeficiency that accompanies AML.

However our data show that T cell activation, indicated by release of IFNγ was not affected by the presence of a dominant AML blast population, though there was a wide variation in the level of IFNγ release between patients. Despite the patients’ quantitative T cell defects (fewer T cells in the PBMC fraction compared to normal PBMC) there was a considerable overlap of the IFNγ levels for patients and controls and there was no significant difference when comparing the AML patient IFNγ output with normal PBMC levels corrected for the difference in normal cell number per culture. Thus, the patients showed a wider range of IFNγ levels than the healthy controls and it will be interesting to determine if this had any effect on prognosis for the patients with high IFNγ levels. As IFNγ is a vital part of the anti-tumor response of the
immune system, it is possible that such patients would benefit from a longer period of remission.

Our studies demonstrated that a population of circulating lymphocytes could be identified by flow cytometry for the majority of patients with untreated AML. The majority of circulating lymphocytes were CD3+T lymphocytes, which were TCRβ+ and usually <10% of the cells were TCRγδ+. The CD4/CD8 T cell ratio varied between patients, but most patients had an excess of CD4+ cells. There were generally very few double positive or double negative T cells. The circulating cells included both CD4+CD45RO+ and CD4+CD45RA+T cells. These characteristics were similar to those reported for AML patients with chemotherapy-induced cytopenia [3]. Interestingly, the distribution of T cell subsets in AML was not significantly different to that seen for the healthy controls, with the exception of the CD8+CD45RA:CD45RO ratio which was significantly lower in the AML patients compared to healthy controls. Whether this reflects increased stimulation of the putative naive pool in these patients to produce memory T cells is one possibility. However, AML is an age-related disease and the expansion of memory T cells with age is now well documented [27]. Although the samples from healthy controls were matched to cover the range of ages seen in AML patients, the group size was much smaller and so we cannot rule out the possibility that the difference in CD45RA vs. CD45RO was due to a predominance of older donors in the AML group.

In our interpretation of the CD45RA/RO data above and the difference between CD8+ cells derived from patients and controls we suggest as one possible explanation that the difference may be caused by stimulation of naive T cells to produce memory cells. However, we would emphasize that this is only one possible explanation; these results should be interpreted with great care and especially with regard to naive and memory T cells. Recent studies using additional membrane molecule markers (i.e. CD62L, CD11a, CD27, CCR7) and the Vβ-chain repertoire have clearly demonstrated that both CD45RA+ and CD45RO+T cell subsets include memory as well as naive T cells, and this is true both in normal individuals and patients [28–30]. Our patients showed a wide variation in the frequency of cells expressing these markers (including double positive cells), and taken together these observations clearly demonstrate that the T cell system shows an extensive variation between AML patients. Additional detailed studies including multiparameter flow cytometry and Vβ repertoire analysis in an even larger group of patients are required to investigate whether the heterogeneous AML patients can be grouped into distinct subsets based on differences in memory and naive T cells, or whether differences in the T cell system correlate to clinical or biological (e.g. genetic abnormalities) characteristics.

PEP005 (ingenol 3-angelate) is a diterpene ester isolated from the plant *Euphorbia peplus*, and this agent appears to have anti-leukemic effects, inducing apoptosis in leukemia cell lines, as well as primary human acute myeloid leukemia (AML) cells [17]. PEP005 is a potent PKC activator [31,32] and its anti-leukemic effects were PKC-δ-dependent [17]. In the present study, we show that PEP005 can induce T cell activation in the presence of human AML cells and significantly increased IFNγ levels were detected for a subset of patients. The divergent effects of this drug on T cell cytokine release is probably caused by a combination of: (i) direct effects on the T cells; and (ii) indirect effects via the AML cells, e.g. variable effects on the constitutive release of immunoregulatory cytokines by the primary AML cells derived from different patients. Further studies are now required to determine if PEP005 may have useful T cell stimulatory effects that could be indirectly advantageous in AML for certain AML patients, in addition to its published direct cytotoxic effects on AML blasts.

In conclusion, our studies demonstrate that the relative frequencies of circulating T cell subsets in patients with untreated AML show similarities to healthy individuals and AML patients with chemotherapy-induced lymphopenia. These T cells can be activated even in the presence of excess AML cells and may thereby represent a target for immunotherapy.

Acknowledgements

The work was supported by the Norwegian Cancer Society and the European commission (LSHB-CT-2004-503467). The helpful flow cytometric assistance of Dr Anita Ryningen and the technical assistance of Kristin Paulsen and Aina Kvinsland are gratefully acknowledged.

References


